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# Characterization and Regulation of the *Pseudomonas aeruginosa* *algC* Gene Encoding Phosphomannomutase\*

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The nucleotide sequence of the *Pseudomonas aeruginosa* *algC* gene encoding phosphomannomutase (PMM; EC 5.4.2.8) was determined. The codon usage in *algC* in the wobble base position was 90.4% G+C, typical of *Pseudomonas* genes. The predicted amino acid sequence of phosphomannomutase (PMM) showed homology over a stretch of 112 amino acids in the carboxyl terminus with rabbit muscle phosphoglucosyltransferase (PGM), an enzyme that catalyzes a reaction analogous to that catalyzed by PMM. In addition, a specific amino acid sequence within PMM showed homology with the catalytic site of PGM. DNA sequence analysis of a defective *algC* gene (*algC'*) cloned from a mutant of *P. aeruginosa* that lacked PMM activity revealed one point mutation (a C to T transition) in the carboxyl terminus of PMM which resulted in an amino acid change from arginine 420 to cysteine 420. The mutation identified in the *algC'* gene was not within the regions of homology with PGM. The *algC* promoter showed significant homology with the promoters of two other *P. aeruginosa* genes involved in alginate synthesis, *algD* and *algR1*. Both the *algD* and *algR1* promoters are activated by the product of the *algR1* gene in *P. aeruginosa*. The upstream region of the *algC* gene contained a sequence identical to the *algD* upstream sequence that is known to be the binding site for the *AlgR1* protein. Expression of *algC* was reduced 5.7-fold in an *algR1* mutant of *P. aeruginosa* compared to its isogenic parent strain (lacking the *algR1* mutation), suggesting that the *algR1* gene product activates the transcription of the *algC* gene.

production of large amounts of hyperviscous bronchial secretions. The accumulation of this material in the respiratory tract of CF patients appears to make these individuals especially vulnerable to bacterial lung infections. *Pseudomonas aeruginosa* is a prevalent pathogen in the lungs of CF patients (1). While *P. aeruginosa* can cause damage to the lung tissue by producing toxins and proteolytic enzymes, the primary complication resulting from *P. aeruginosa* infection in CF is the production by the bacterial cells of a slimy exopolysaccharide known as alginate (1). The presence of alginate exacerbates the respiratory difficulties resulting from the abnormally viscous CF lung environment.

The alginate layer surrounding *P. aeruginosa* in the CF respiratory tract provides a protective barrier against antibiotics and the host immune defenses (1, 2). Clearly, prevention of alginate synthesis by *P. aeruginosa* in the CF lung would enhance existing treatment strategies. Thus, compounds that inhibit enzymes required for alginate synthesis in *P. aeruginosa* (see Fig. 1) have potential use as therapeutic agents. The development of such drugs has been hindered by the inability to obtain sufficient quantities of alginate biosynthetic enzymes for characterization and inhibitor studies. This is due to the extremely low activity levels of these enzymes in cell-free extracts, even in those prepared from heavily mucoid (alginate-producing) *P. aeruginosa* strains (3). To circumvent this problem, many of the *P. aeruginosa* alginate (*alg*) genes have been cloned in broad host range-controlled expression vectors to allow overproduction of the gene product of interest. Two alginate biosynthetic enzymes from *P. aeruginosa*, PMI-GMP (the *algA* gene product), and GMD (the *algD* gene product) (see Fig. 1), have been overproduced and purified using this method (4-7). The gene encoding PMM (step 2, Fig. 1), was of interest as PMM represents yet another potential target of inhibition of alginate synthesis. In addition, although phosphomannomutases from yeast (8, 9) and plants (10) have been studied, very little information about PMM from bacterial sources has been reported in the literature. In this paper, we describe the cloning of the *P. aeruginosa* *algC* gene encoding PMM, present the nucleotide sequences of the wild-type and mutant *algC* genes as well as the transcriptional and translational initiation sites of the wild-type gene, and examine the transcriptional regulation of the *algC* gene in *P. aeruginosa*.

One clinical manifestation of cystic fibrosis (CF)<sup>1</sup> is the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M60873.

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The abbreviations used are: CF, cystic fibrosis; PMM, phosphomannomutase; PMI-GMP, phosphomannose isomerase-GDP manose pyrophosphorylase; GMD, guanosine diphospho-D-mannose dehydrogenase; Ap, ampicillin; Km, kanamycin; Tc, tetracycline; bp, base pair; kb, kilobase pair; EMS, ethyl methane sulfonate; PIA, *Pseudomonas* isolation agar; IPTG, isopropyl β-D-thiogalactopyranoside; MOPS, N-morpholinopropanesulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; PGM, phosphoglucosyltransferase.

## EXPERIMENTAL PROCEDURES<sup>2</sup>

### RESULTS

*Cloning of a P. aeruginosa* Gene Encoding PMM—The activity levels of PMM and other alginate biosynthetic en-

<sup>1</sup> Portions of this paper (including "Experimental Procedures," part of "Results," Tables I-III, and V, and Figs. 3-9) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

zymes in crude extracts of *P. aeruginosa* are extremely low and can barely be detected (3). One of our objectives was to clone the *P. aeruginosa* *algC* gene under *tac* promoter control to allow overproduction of PMM, thereby obtaining sufficient amounts of the enzyme for characterization and inhibitor studies. In order to achieve this objective, we first identified an alginate-negative mutant of *P. aeruginosa* that lacked PMM activity (strain 8858), using the strategy outlined under "Results" in the Miniprint. We then screened genomic libraries of *P. aeruginosa* 8830 for recombinant plasmids that would complement the *alg-28* mutation in mutant 8858, using the approach described under "Experimental Procedures." From a *Bam*HI-generated library, we recovered a recombinant plasmid (pAB8) that restored the mucoid (alginate-producing) phenotype to mutant 8858. The level of alginate produced by strain 8858 containing pAB8 was comparable to that observed for the mucoid parent strain 8830 (Table III).

Subcloning of various cloned fragments from within the 26-kb insert in pAB8 localized the ability to complement mutant 8858 back to the mucoid phenotype to a 2.6-kb *Hind*III-*Sst*I fragment. The plasmid containing this 2.6-kb fragment was designated pNZ15. The size of the 2.6-kb insert was further decreased by making unidirectional deletions from the *Hind*III end using exonuclease III. This resulted in a 1.8-kb fragment that complemented mutant 8858 but only when cloned downstream of a vector-encoded promoter sequence (pNZ15-Δ2C). All of the various plasmids that contained subcloned fragments of the original 26-kb insert in pAB8 and still complemented mutant 8858 were passaged through *Escherichia coli* AC80. Upon reintroduction into 8858 these plasmids again restored the mucoid phenotype. We demonstrated previously (29) by hybridization analysis that the cloned 2.6-kb insert in pNZ15 was of *P. aeruginosa* origin. This fragment hybridized with a 2.6-kb fragment of *Hind*III-*Sst*I-digested chromosomal DNA from *P. aeruginosa* strains 8830 and PAO1, as well as with DNA fragments of varying sizes from several other *Pseudomonas* species belonging to *Pseudomonas* rRNA homology group I, *Azomonas macrocytogenes*, *Azotobacter vinelandii*, *Serpens flexibilis*, and *Xanthomonas campestris* (29). The fragment did not hybridize with *Hind*III-*Sst*I-digested chromosomal DNA from *E. coli*, *Salmonella typhimurium*, or *Klebsiella pneumoniae* (29).

**Identification of *P. aeruginosa* Proteins Encoded by the 2.6-kb Insert in Plasmid pNZ15**—In order to determine the number and size(s) of polypeptides encoded by the cloned *P. aeruginosa* DNA in plasmid pNZ15, and the direction of transcription of the putative *P. aeruginosa* *algC* gene, the 2.6-kb *Hind*III-*Sst*I fragment was cloned into the vectors pT7-5 and pT7-6 (16, 17). This allowed exclusive labeling of the plasmid-encoded polypeptides using a T7 RNA polymerase/plasmid promoter system in *E. coli* strain K-38/pGP1-2 (16, 17) (see "Experimental Procedures"). The resulting hybrid plasmids, pNZ530 and pNZ631, contain the cloned *Hind*III-*Sst*I fragment of *P. aeruginosa* DNA in opposite orientations

relative to the T7 RNA polymerase promoter (Fig. 2). In these vectors, the  $\beta$ -lactamase gene is not selectively transcribed. The plasmids were introduced into *E. coli* strain K-38/pGP1-2 and plasmid-encoded proteins were selectively labeled with [<sup>35</sup>S]methionine following growth, heat induction, and rifampicin treatment of the strains (16, 17). The results shown in Fig. 2 indicate that a single polypeptide having an estimated molecular weight of 50,000 was synthesized from the cloned insert in plasmid pNZ631. This polypeptide was formed even in the absence of thermal induction, demonstrating incomplete repression of the T7 RNA polymerase at 30 °C in these strains. No additional polypeptides were synthesized in plasmid pNZ530 compared to the vector control pT7-5, suggesting that the direction of transcription of the putative *algC* gene is from *Hind*III to *Sst*I in the 2.6-kb cloned insert in pNZ15.

**Enzymological Analyses**—Plasmids containing the putative *P. aeruginosa* *algC* gene, either on the aforementioned 2.6-kb *Hind*III-*Sst*I fragment or its 1.8-kb deletion derivative were introduced into strain 8858 to test for increased PMM activity (Table IV). Strain 8858 alone had extremely low levels of PMI and PMM activity. The presence of plasmid pNZ15 in strain 8858 led to a high level of PMM activity. When the

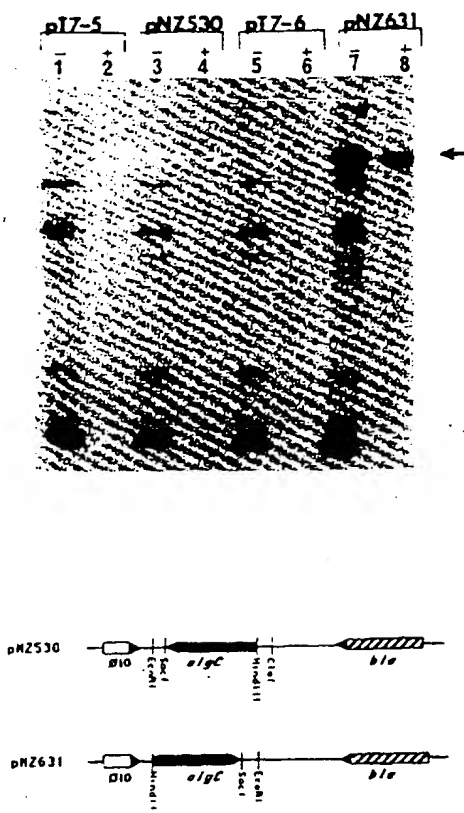


FIG. 2. Autoradiogram showing the single polypeptide encoded by the cloned 2.6-kb *Hind*III-*Sst*I DNA fragment that complements *P. aeruginosa* mutant 8858. A + indicates thermal induction (42 °C for 10 min). A - indicates no induction. Cells were treated with rifampicin prior to <sup>35</sup>S-labeling of proteins. After SDS-PAGE, radioactive polypeptides were visualized by autoradiography. Lanes 1 and 2, pT7-5 vector control; lanes 3 and 4, pNZ530 (2.6-kb *Hind*III-*Sst*I fragment cloned into pT7-5 with the *Sst*I site proximal to the T7 promoter); lanes 5 and 6, pT7-6 vector control; lanes 7 and 8, pNZ631 (2.6-kb *Hind*III-*Sst*I fragment cloned into pT7-6 with the *Hind*III site proximal to the T7 promoter). The position of PMM is indicated by the arrow. The position of the cloned inserts relative to the T7 promoter in each plasmid is represented schematically in the bottom of the figure.

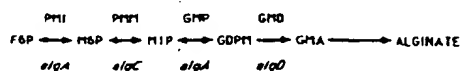


FIG. 1. Alginate biosynthetic pathway in *P. aeruginosa*. Abbreviations: F6P, fructose 6-phosphate; M6P, mannose 6-phosphate; M1P, mannose 1-phosphate; GDPMP, GDP-mannose; GMA, GDP-mannuronic acid; PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP, GDP-mannose pyrophosphorylase; GMD, GDP-mannose dehydrogenase. The *P. aeruginosa* *algA* gene encodes a bifunctional PMI-GMP (7), *algD* encodes GMD (4), and *algC* encodes PMM (this report). Steps between GMA and alginate include polymerization, epimerization, acetylation, and export, although little information is currently available about these steps.

TABLE IV  
Specific activities of PMI and PMM in *P. aeruginosa* 8858 and *E. coli* AC80 containing subclones derived from pAB8

Strain/plasmid*	Specific activity			
	PMI		PMM	
	-IPTG	+IPTG	-IPTG	+IPTG
milliunits/mg				
<i>P. aeruginosa</i>				
8858	<1.0 <sup>a</sup>	<1.0	<1.0 <sup>a</sup>	<1.0
8858/pNZ15	<1.0	NT <sup>b</sup>	93.6	NT
8858/pMMB22	<1.0	<1.0	<1.0	<1.0
8858/pNZ18	<1.0	<1.0	54.7	88.2
8858/pMMB66HE	<1.0	<1.0	<1.0	<1.0
8858/pNZ48	<1.0	<1.0	51.9	38.8
8858/pNZ49	<1.0	<1.0	156.3	325.0
<i>E. coli</i>				
AC80	202.4 <sup>b</sup>	NT	87.9 <sup>b</sup>	NT
AC80/pMMB66HE	225.1	170.5	86.3	NT
AC80/pNZ49	172.2	239.6	76.4	76.2

\* Plasmid pNZ15 contains the *algC* gene cloned as a 2.6-kb fragment in pJRD215 (23). Plasmid pNZ18 contains the same 2.6-kb fragment cloned under *tac* promoter control in plasmid pMMB22 (44). Plasmids pNZ48 and pNZ49 contain the *algC* gene (cloned as a 1.8-kb fragment under *tac* promoter control) in plasmids pMMB66EH and pMMB66HE (30), respectively.

<sup>a</sup> A specific activity of <1.0 for PMI represents the wild-type level for *P. aeruginosa* (3). The endogenous levels of PMI and PMM activity shown for *E. coli* AC80 are typical for this strain grown under these conditions (3).

<sup>b</sup> Not tested as the *algC* gene is not under *tac* promoter control.

2.6-kb *HindIII*-*SstI* fragment was cloned in the proper orientation under *tac* promoter control (plasmid pNZ18), a high level of PMM activity was also observed. This activity did not exceed that obtained with plasmid pNZ15, and the activity increased only moderately upon IPTG induction (Table IV). DNA sequencing of the 2.6-kb fragment cloned in pNZ15 (see below) indicated that the single open reading frame in this fragment lies approximately 1.2 kb from the *HindIII* end of the fragment. Thus, it appeared that the inability to overexpress the putative *algC* gene in pNZ18 was due to the 1.2 kb of intervening DNA between the *tac* promoter and the start of the *algC* gene. To test this possibility, the 1.8-kb deletion derivative of the 2.6-kb *HindIII*-*SstI* fragment was cloned in both orientations relative to the *tac* promoter in the vectors pMMB66EH and pMMB66HE (30) to create plasmids pNZ48 and pNZ49, respectively. The latter plasmids were introduced into *P. aeruginosa* strain 8858 and crude extracts were prepared from cultures grown with and without IPTG induction (Table IV). Although a high level of PMM activity was observed in 8858 containing pNZ48, the activity did not exceed that observed for plasmid pNZ18, and the PMM activity did not increase upon IPTG induction. Strain 8858/pNZ49 yielded the highest PMM activity observed for any strain/plasmid combination, and this PMM activity increased 2.1-fold upon IPTG induction. The direction of transcription of the putative *algC* gene relative to the *tac* promoter in pNZ49 was the same as that in plasmid pNZ631, the only construct that directed the synthesis of a <sup>35</sup>S-labeled protein using the T7 RNA polymerase/plasmid promoter system (see Fig. 2). No increase in PMI activity was observed in any *P. aeruginosa* strain harboring a plasmid containing the putative *algC* gene, and no PMM (or PMI) activity was found associated with the plasmid vectors alone (Table IV).

*E. coli* contains high endogenous levels of PMI and PMM activity (3, Table IV). Introduction of plasmid pNZ49 did not lead to a significant difference in the level of PMM (or PMI) activity in crude extracts of *E. coli*, even upon IPTG induction

(Table IV). However, when a crude extract of *E. coli* AC80/pNZ49 (prepared from IPTG-induced cells) was fractionated by ion-exchange chromatography, a second peak of PMM activity was observed in addition to the peak of PMM activity endogenous to *E. coli* (Fig. 3).

Samples of the crude extracts prepared from *P. aeruginosa* 8858 and *E. coli* AC80 overexpressing the putative *P. aeruginosa* *algC* gene, along with the appropriate vector and uninduced controls, were subjected to SDS-PAGE (Fig. 4). The *P. aeruginosa* strains having elevated PMM activity, 8858/pNZ18 and 8858/pNZ49, contained a protein band corresponding to an estimated molecular weight of 51,000. This was in agreement with the molecular weight (50,000) of the single <sup>35</sup>S-labeled polypeptide synthesized from the 2.6-kb *HindIII*-*SstI* fragment in plasmid pNZ631 (Fig. 2). *E. coli* contained numerous intense protein bands that comigrated to the position corresponding to a molecular weight of 51,000 (see Fig. 4). Thus, it was not possible to discern an additional band encoded by the putative *P. aeruginosa* *algC* gene in *E. coli* AC80/pNZ49 even with IPTG induction. Likewise, when samples of column fractions spanning both peaks of PMM activity from *E. coli* AC80/pNZ49 (+IPTG) recovered from ion-exchange chromatography (see Fig. 3) were subjected to SDS-PAGE, numerous intense protein bands in the *M*, 51,000 range obscured any band that may have been encoded by the cloned insert in pNZ49 (data not shown).

To confirm that the cloned DNA fragment in pNZ49 contained the *algC* structural gene encoding the PMM polypeptide, PMM from *P. aeruginosa* 8858/pNZ49 was purified to an extent that allowed its N-terminal amino acid sequence to be determined (see "Experimental Procedures"). Following sequential purification steps using ion-exchange, hydroxylapatite, and gel-filtration chromatography (Fig. 5), PMM was judged to be >90% pure based on SDS-PAGE (Fig. 6). The N-terminal amino acid sequence of the protein, read to 19 amino acids, was S-T-V-K-A-P-T-L-P-A-S-I-F-R-A-Y-D-I-R. This sequence corresponded exactly to the N-terminal amino acid sequence predicted from the nucleotide sequence of the *algC* gene (described below).

**DNA Sequence Analysis**—The complete nucleotide sequence of the *P. aeruginosa* *algC* structural gene contained within the 1.8-kb DNA fragment that complemented mutant 8858 (described above) was determined (Fig. 7). One open reading frame was identified which was capable of coding for a protein of 463 amino acids. The calculated molecular weight of PMM predicted from the nucleotide sequence of the *algC* gene (50,269) was in agreement with the molecular weight of PMM (51,000) determined by SDS-PAGE. The codon usage in the wobble base position was 90.4% G+C (see Table V), typical of *Pseudomonas* genes (31, 32). The direction of transcription of *algC* was shown (Fig. 2) to be from the *HindIII* site to the *SstI* site of the 2.6-kb fragment in plasmid pNZ15. Following determination of the N-terminal amino acid sequence of PMM (described above), it was determined that translation of the *algC* message initiated with a methionine and terminated with a TGA codon 1,388 nucleotides downstream of the ATG (Fig. 7). A putative ribosome-binding sequence, GGAG (33), was located 12 bp upstream of the translational initiation codon (Fig. 7). The 5' end of the *algC* mRNA was found to be a G located 244 bp upstream of the translational initiation site (data shown below).

A sequencing strategy analogous to that used for the wild-type *algC* gene was used to sequence the *algC'* gene cloned from the PMM<sup>-</sup> mutant *P. aeruginosa* 8858 (as described under "Experimental Procedures"). A single C to T transition was found at nucleotide position 1505 of the *algC'* coding

region, resulting in a change from arginine 420 to cysteine 420 (data not shown).

Comparison of the nucleotide sequence of *algC* with other nucleotide sequences in GenBank®/EMBL Data Bank (34) showed only weak overall homologies. However, PMM did show significant localized amino acid homology with phosphoglucosyltransferase (PGM) from rabbit muscle (35). PMM and PGM catalyze analogous interconversions of hexose 6-phosphates and hexose 1-phosphates. The 44% sequence similarity was restricted to a 112 amino acid stretch located in the carboxyl terminus of both enzymes (Fig. 8). The significance of the homology in this region is not known at present.

PGM belongs to a family of phosphoserine enzymes which share a serine residue that is phosphorylated in the active form of the enzyme (36). The amino acid sequence Thr-Ala-Ser-His-Asn is known to be critical for PGM activity, and this sequence is within the 21 amino acid long active site region of PGM (35). The amino acid sequence Thr-Gly-Ser-His-Asn was found in the *algC* coding sequence (amino acid positions 106–110, Fig. 7). The region surrounding the Thr-Gly-Ser-His-Asn sequence in PMM was compared with the 21 amino acid long active site region of PGM (Fig. 9). Considerable homology (57%) between PMM and PGM was observed in this region (which lies in the N-terminal portion of both proteins). Furthermore, of the matched amino acids comprising this 57% homology, 75% (9 out of 12) were exact matches (only 25% of the matches were conserved replacements, see Fig. 9).

#### Identification of the Transcriptional Initiation Site of *algC*

The 5' end of the *algC* gene was mapped using S1 nuclease protection (24). A 15-bp synthetic oligonucleotide (5'-GCG-GAAGATGCTGGC3') designated PE1 was used as primer. PE1 is complementary to nucleotides 275–290 of the *algC* DNA sequence (Fig. 7). A 1.5-kb *HindIII*-*XhoI* DNA fragment from within the 2.6-kb DNA fragment in plasmid pNZ15 contains the putative *algC* promoter region and an additional 1 kb of upstream *P. aeruginosa* DNA. This fragment was cloned into the *HindIII*-*SalI* sites of M13mp19 resulting in phage mNZ25 which was used as the source of single-stranded DNA. The 1.5-kb *HindIII*-*XhoI* fragment was hybridized to total cellular RNA from the mucoid *P. aeruginosa* strain 8830 and to RNA from a nonmucoid strain of *P. aeruginosa* (8822) harboring plasmid pNZ15-ΔHE, which contains the *algC* structural gene and 1.2 kb of *P. aeruginosa* DNA upstream of the translational start site of *algC* (the promoter of the vector-encoded tetracycline resistance gene was deleted in plasmid pNZ15-ΔHE, see below). Analysis of the protected DNA fragment(s) on a low resolution polyacrylamide gel revealed one strong signal migrating to a position corresponding to 274 bp in length (Fig. 10). Since the same primer was used for the S1 mapping and the dideoxy sequence analysis, transcription was determined to initiate with a guanosine located 244 bp upstream of the *algC* translational initiation codon.

A comparison of the upstream region preceding the *algC* structural gene with the consensus *E. coli* RNA polymerase  $\sigma^{70}$  recognition sequence revealed no similarity in the –10 or the –35 regions. We did observe that the GC at positions –21 and –22 and the GG at positions –33 and –34 of the *algC* gene had the same spacing as the GC at positions –12 and –13 and the GG at positions –24 and –25 of an enteric bacterial *rpoN* ( $\sigma^{54}$ ) consensus sequence (37, 38) (Fig. 11). However, the position of the bases relative to the transcriptional start site was different in the *algC* sequence and the *rpoN* consensus sequence. The significance of this shift in position is not known at present. More importantly, perhaps, was the finding that the GC and GG sequences at positions

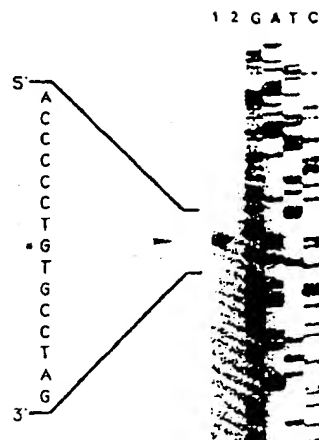


FIG. 10. Identification of the transcriptional initiation site of *algC*. S1 nuclease mapping was performed with a 15-mer synthetic oligonucleotide (PE1) which was complementary to the sense strand of the 5' end of *algC*. The probe was hybridized to total cellular RNA from *P. aeruginosa* strain 8830 (lane 1) and *P. aeruginosa* strain 8822 harboring the plasmid pNZ15-ΔHE (lane 2). Products of the S1 nuclease reactions are adjacent to dideoxy sequencing reaction lanes (GATC) prepared with primer PE1. The complementary sequence is shown to the left of the sequencing reaction. The arrow and asterisk indicate the guanosine corresponding to the products protected from S1 nuclease. This position is 244 bp upstream of the translational start site (see Fig. 7).

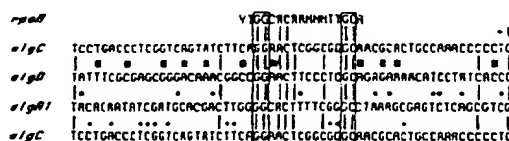


FIG. 11. Comparison of the sequence of the putative promoter region of *algC* with that of *algD*, *algR1*, and the  $\sigma^{54}$  (*RpoN*) consensus sequence. The transcriptional start site is designated as +1. The GG and GC within the putative  $\sigma^{54}$  recognition sequence are boxed. Identical base matches between *algC* with *algD*, *algR1*, and the *rpoN* consensus are indicated by solid vertical lines. Identical bases between *algC* and *algD* are indicated by ■, between *algD* and *algR1* by ●, and between *algR1* and *algC* by ♦. Other letters are defined as follows: Y, pyrimidine; R, purine; and N, any base.

–21/–22 and –33/–34 of *algC* also exist at these same positions in the upstream region of two other *P. aeruginosa* genes involved in alginate synthesis, *algD* and *algR1* (39, 40) (Fig. 11).

**Analysis of *algC* Gene Expression**—To construct plasmid pNZ15, the 2.6-kb *HindIII*-*SstI* fragment containing the *algC* structural gene was cloned between the *HindIII*-*SstI* sites of pJRD215, which placed the *algC* gene downstream of the tetracycline resistance gene promoter present in pJRD215 (23). To determine if the *algC* gene was being expressed from this vector-specific promoter or from its own promoter, the *EcoRI*-*HindIII* vector segment containing the *tet* promoter was removed from pNZ15, generating plasmid pNZ15-ΔHE. This construct was still able to complement the alginate-negative *P. aeruginosa* mutant 8858, indicating that the *algC* gene within the 2.6-kb fragment was indeed being expressed from its own promoter.

The *P. aeruginosa* *algD* gene is transcriptionally activated in mucoid strains of *P. aeruginosa* (4). This activation is mediated by the product of the *algR1* gene, a *trans*-acting regulatory protein that autoregulates the expression of *algR1* itself as well as activating *algD* (40). Since the upstream region of the *algC* gene showed similarity both in sequence and spacing to the upstream regions of both the *algD* and *algR1*

TABLE VI  
 $\beta$ -Galactosidase activities in *P. aeruginosa* strains containing the *algC-lacZ* transcriptional fusion

Strain/plasmid <sup>a</sup>	$\beta$ -Galactosidase specific activity <sup>b</sup>
8830/pNZ63	51
8852/pNZ63	9
8858/pNZ63	200

<sup>a</sup> Cells were grown for 18 h in YTG medium (see "Experimental Procedures"). The doubling time (2 h) was the same for all strains in this medium.

<sup>b</sup> Specific activities are defined as nanomoles of *o*-nitrophenol formed/minute/milligram of crude extract protein. The specific activity values given are the average of three independent experiments that gave essentially identical results.

genes (Fig. 11), it was possible that the *algC* gene was also transcriptionally regulated by the *algR1* gene product. To test this possibility, a transcriptional fusion vector was constructed using the *algC* upstream region from pNZ15 (1.5 kb of DNA upstream of the internal *XhoI* site at nucleotide position 533, see Fig. 7) and the promoterless *lacZ* structural gene in the vector pKRZ-1, a broad host-range promoter probe vector based on the pSA origin of replication.<sup>3</sup> This placed the promoterless *lacZ* gene directly under the control of the *algC* promoter sequence and 1.0 kb of upstream DNA, creating plasmid pNZ63. Plasmid pNZ63 was introduced into the stable mucoid (alginate-producing) *P. aeruginosa* strain 8830 and an *algR1* mutant derived from 8830 (strain 8852), to determine if transcriptional activation of *algC* was dependent on a functional *algR1* gene. *E. coli* CSH50 (25) containing pNZ63 was also examined as a negative control. In three independent experiments, the specific activity of  $\beta$ -galactosidase was 4.8–6.6-fold (average 5.7-fold) lower in 8852/pNZ63 compared to 8830/pNZ63 grown under the same conditions (Table VI). Negligible levels of  $\beta$ -galactosidase activity were detected in *E. coli* CSH50/pNZ63.

PMM activity is induced in *P. aeruginosa* in response to overproduction of the preceding enzyme of the alginate biosynthetic pathway, phosphomannose isomerase (3). This suggested that the product of the PMI reaction, mannose 6-phosphate, may be involved in the induction of PMM activity. Since *P. aeruginosa* strain 8858 lacks PMM activity, it would be expected to accumulate mannose 6-phosphate as a result of the mutational block at PMM. If mannose 6-phosphate was indeed involved in inducing PMM activity by increasing the level of *algC* transcription, then strain 8858 should have high levels of *algC* transcription, even though the message formed coded for an inactive PMM. To test this possibility, we introduced the *algC-lacZ* fusion vector pNZ63 into strain 8858 and compared the level of  $\beta$ -galactosidase activity to strain 8830 (from which 8858 was derived) (11) containing pNZ63. The level of *algC* transcription, measured as  $\beta$ -galactosidase activity, was 4-fold higher in strain 8858/pNZ63 than in 8830/pNZ63 (Table VI).

#### DISCUSSION

The inherently low activity levels of alginate biosynthetic enzymes in mucoid strains of *P. aeruginosa* (3, 41) have made it difficult to attribute the loss of alginate production in Alg<sup>−</sup> mutants to a loss of a specific enzymatic activity. Also, such low enzyme activities hinder the purification of sufficient quantities of enzymes needed for rigorous characterization for development of inhibitors that have potential therapeutic application in the treatment of *P. aeruginosa* respiratory tract

infections in CF patients. Our laboratory successfully cloned and overexpressed two *P. aeruginosa* *alg* genes, *algA* and *algD*, and overproduced and purified their respective gene products, PMI-GMP and GMD (4–7). In this report we describe the cloning, sequencing, and genetic characterization of yet another *P. aeruginosa* *alg* gene, *algC* (encoding PMM), and examine its transcriptional regulation.

We used an indirect approach to identify an Alg<sup>−</sup> mutant of *P. aeruginosa* that lacked PMM activity. Sa'Correia *et al.* (3) showed that overproduction of PMI in *P. aeruginosa* led to a simultaneous increase in PMM activity. By overexpressing the *algA* gene in several Alg<sup>−</sup> mutants of *P. aeruginosa*, we were able to show that strain 8858 (*his-1, alg-28*) lacked the increase in PMM activity that normally accompanies overproduction of PMI, and thus appeared to have a mutation in the *algC* structural gene. Wang *et al.* (28) showed that none of the *alg* genes cloned previously in this laboratory encoded PMM, and it is interesting that strain 8858 belonged to a group of Alg<sup>−</sup> mutants that were not complemented by any of the cloned *alg* genes in our collection. It was possible, however, that the mutation in strain 8858 resided in a regulatory gene that was involved in the induction of PMM activity. The loss of such a regulatory gene would be manifested in *P. aeruginosa* as an Alg<sup>−</sup> phenotype and in an absence of elevated PMM activity in response to overproduction of PMI, characteristics indistinguishable from an *algC* structural gene mutant.

Strong evidence in favor of strain 8858 containing a mutation in the *algC* structural gene came from cloning of a *P. aeruginosa* gene that complemented the *alg-28* mutation and restored the mucoid phenotype to strain 8858. Plasmid pNZ49 contained the putative *algC* gene cloned under control of the *tac* promoter. Introduction of pNZ49 into strain 8858 led to highly elevated PMM activity that increased still further (2.1-fold) upon IPTG induction (Table IV). In addition, when plasmid pNZ49 was introduced into *E. coli* AC80 and the putative *algC* gene overexpressed by IPTG induction, two peaks of PMM activity were resolved following ion-exchange chromatography of crude extracts. Only the larger trailing peak of PMM activity shown in Fig. 3 is normally present in extracts of *E. coli* AC80. It is very unlikely that a *P. aeruginosa* regulatory gene that induces PMM activity in *P. aeruginosa* could cause induction of a previously unrecognized second isozyme of PMM in *E. coli*.

Taken together, these data suggested that the gene cloned in pNZ49 was the *P. aeruginosa* *algC* structural gene encoding PMM. However, proof of this could only be obtained by determining the N-terminal amino acid sequence of purified PMM and comparing it to the N-terminal amino acid sequence predicted from the nucleotide sequence of the *algC* gene. We have confirmed that the cloned gene in pNZ49 does in fact encode PMM, based on 100% agreement between the first 19 N-terminal amino acids in the purified PMM and the N-terminal sequence predicted from the nucleotide sequence of the *algC* gene. To our knowledge this is the first report of the cloning and sequencing of a gene encoding PMM from any organism.

The sequence similarity between the 21 amino acid long active site region in the N-terminal portion of PGM (35) and the region surrounding the amino acid sequence Thr-Gly-Ser-His-Asn in the N-terminal end of PMM (Fig. 9) suggests that this region may contain the PMM active site. Furthermore, the similarity in this region of the two proteins suggests that in PMM, like PGM (36), the serine residue within the active site may need to be phosphorylated to produce an active enzyme. Investigations into that possibility are underway using oligonucleotide-directed site-specific mutagenesis to

<sup>3</sup> R. Rothmel, manuscript in preparation.



identify regions of the *algC* gene that are important in forming the active site of PMM. The significance of the 44% sequence similarity in the carboxyl end of PMM and PGM is not known at present as the functional role of this region in either protein, if indeed one exists, is not known.

The nucleotide sequence of a mutant *algC* gene (*algC'*) cloned from the PMM<sup>-</sup> mutant *P. aeruginosa* 8858 was also determined. One point mutation (a C to T transition) was detected at nucleotide position 1505, which results in an amino acid change of arginine 420 to cysteine 420. Both the *algC* and *algC'* genes were cloned behind the *tac* promoter in the proper orientation and overexpressed in *P. aeruginosa* 8858. Analysis by SDS-PAGE showed that both extracts contained one overproduced protein band corresponding to a subunit molecular weight of 51,000, the size of the wild-type PMM.<sup>4</sup> Since DNA sequencing of the *algC'* gene did not reveal any nonsense mutations, and since the *algC* and *algC'* genes appear to code for proteins of the same molecular weight, we conclude that the *algC'* gene cloned from the PMM<sup>-</sup> mutant 8858 does not encode a truncated PMM. The mutation in the *algC'* gene is not within the putative active site sequence of PMM (Fig. 9) or in the region of homology with PGM (Fig. 8). Thus, the mechanism by which the arginine 420 to cysteine 420 mutation in the *algC'* gene abolishes PMM activity in mutant 8858 is not known at present.

Once the transcriptional initiation site for the *P. aeruginosa* *algC* gene was determined, the promoter region was compared to other prokaryotic promoters to determine which nucleotides may be important in RNA polymerase recognition. We found no sequence in the *algC* promoter region that resembled the *E. coli* consensus  $\sigma^{70}$  promoter sequence. This may explain the absence of  $\beta$ -galactosidase activity when pNZ63 (the *algC* promoter-*lacZ* transcriptional fusion vector) was present in *E. coli* CSH50. Although we did observe that the GC and the GG at positions -21/-22 and -33/-34, respectively, of the *algC* upstream region had the same spacing as the GC (-12/-13) and GG (-24/-25) of an *rpoN* consensus sequence, the difference in position and intervening sequence (see Fig. 11) makes it impossible to make any conclusions with respect to a possible functional parallel between the two promoters. However, the considerable homology between the *algC* promoter and the promoter sequences of two other *P. aeruginosa* genes involved in alginate synthesis, *algD* and *algR1* (see Fig. 11) is worthy of mention. The 1.8-kb DNA fragment that contains the entire *algC* coding region, the transcriptional initiation site, and 67 bases upstream of the *algC* transcriptional initiation site does not complement the PMM<sup>-</sup> mutant 8858 unless cloned downstream of a plasmid promoter sequence. This suggests that there are bases further upstream of the putative *algC* promoter sequence that are crucial for initiating transcription. Both the *algD* and *algR1* genes of *P. aeruginosa* are activated by the *algR1* gene product AlgR1 (40), and AlgR1 has recently been demonstrated by DNA footprinting experiments to bind at the -376 to -389 and -452 to -465 14-mer sites having the sequence CCGTTTCGTCN<sub>3</sub>. This sequence is upstream of the *algD* transcriptional initiation site. This far upstream sequence is essential for activation of the *algD* promoter (42). Interestingly, the sequence CCGTTTCGTC was also found in the upstream region of *algC* (nucleotides -86 to -94, see Fig. 7). The presence of this sequence, together with the homology between the putative promoter of the *algC* gene and the *algD* and *algR1* promoters, suggested that transcription of *algC*, *algD*, and *algR1* may be controlled by similar regulatory mechanisms, requiring *trans*-activation by AlgR1. We tested

this possibility by introducing the *algC-lacZ* transcriptional fusion vector pNZ63 into the mucoid *P. aeruginosa* strain 8830 and its isogenic *algR1* mutant strain 8852 (40). The 5.7-fold lower level of  $\beta$ -galactosidase activity in 8852/pNZ63 compared to 8830/pNZ63 (Table VI) suggested that the *algR1* gene product does in fact play a role in the transcriptional regulation of *algC*. It is noteworthy that AlgR1 activates at least two alginate genes mapping at different positions on the *P. aeruginosa* genome; *algD* at 34 min (40) and *algC* (map position unknown but distinct from *algD*), and that the consensus AlgR1-binding sequence CCGTTTCGTCN<sub>3</sub> has been located in the far upstream region of both of these genes. Further experiments are in progress to elucidate the regulatory mechanisms governing *algC* gene expression and to determine the overall role of *algR1* in regulating alginate biosynthesis in *P. aeruginosa*.

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PPM was purified for N-terminal amino acid sequence determination from IPTG-induced cultures of *E. aeruginosa* 0040/pa289.

Phosphomannomutase from *Pseudomonas*

Ion-exchange chromatography. Fifty mg of crude extract protein prepared in buffer A as described above in a volume of 3 ml was fractionated on Pharmacia FPLC chromatography system using a Mono Q, HQ 10/10 column. The Pharmacia FPLC chromatography system using a Mono Q, HQ 10/10 column. The starting buffer was buffer A. The flow rate was 2 ml/min and 2 ml fractions were collected. Following sample application, unbound material was washed through the column with 40 ml of buffer A. Bound material was then eluted with 0.3 M of a linear gradient of NaCl (0-0.3 M) followed by 20 ml of 1.0 M NaCl. All NaCl solutions were prepared in buffer A.

**Hydroxyapatite chromatography.** Fractions 28-31 from the ion-exchange step were pooled and concentrated by ultrafiltration. The sample was then applied to a column of Sephadex 2-25 (Pharmacia 100 cm column) using 10 mM potassium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 10% glycerol for elution and elution. The sample was then applied to a column of hydroxyapatite (Bio-Gel HTP) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 10% glycerol. The flow rate was 2 ml/min and 2 ml fractions were collected. Fractions 1-12 were pooled and concentrated by ultrafiltration. Fractions 13-17 were pooled and concentrated to 0.3 ml by ultrafiltration.

[illegible]

Crude extracts of *E. coli* AC80 and AC80/pHE49 (prepared from PTC-induced cells) were fractionated by ion-exchange chromatography using exactly the same procedure as outlined above for the initial purification step for the *E. spruginosa* PPM.

### Quantitation of Aldehyde Production

2. *Escherichia* strains were grown as confluent lawns on FIA [containing tetracycline (if needed). Cells were scraped from the plates at the indicated times and suspended in 3 ml of sterile 0.9% saline. 2 ml of the suspension was centrifuged at 20,000 x g for 10 min. The pellet was washed with distilled water. The supernatant was removed and the cells were resuspended in 1 ml of distilled water. The uronic acid concentration of the dialyzed sample was then determined by the method of Knutton and Jeenes (17). The cells were washed with distilled water and 2 ml of cell suspension was resuspended in 1 ml H<sub>2</sub>O. To 0.6 ml of the suspension, 0.2 ml of 25% trichloroacetic acid was added to lyse the cells. The suspension was allowed to stand at room temperature for 10 min. The suspension was then centrifuged at 20,000 x g for 10 min. The supernatant was removed and the pellet was then resuspended in 0.2 ml of 50 mM potassium phosphate buffer (pH 7.0) and the protein concentration determined. Alginate production is expressed as mg alginate per mg protein from 2 ml of

## نتیجه‌گیری

[illegible]

TABLE I.

Strain or plasmid	Genotype or relevant characteristic	Source or reference
<b><i>Escherichia coli</i></b>		
AC80	str lsm met nad <sup>+</sup> nad <sup>+</sup>	(43)
HM101	l <sup>+</sup> supD20 (l <sup>+</sup> ) (recA) ara-14 proA <sup>+</sup> lacY <sup>+</sup> galK <sup>+</sup> proU <sup>+</sup> xylX <sup>+</sup> str <sup>r</sup> supE <sup>+</sup> (Str <sup>R</sup> )	(15)
JM109	$\Delta$ (lacZ-lacI) recA1 thi-1 supE <sup>+</sup> ara <sup>+</sup> xylX <sup>+</sup> nad <sup>+</sup> trp <sup>+</sup> l <sup>+</sup> trcA16 proA <sup>+</sup> lacI <sup>+</sup> lacO <sup>+</sup> (5)	(21)
K-18	host strain for T7 RNA polymerase-lac <sup>+</sup> promoter system	(16)
CSH50	str Prot <sup>+</sup> ara <sup>+</sup> (ara <sup>+</sup> -lac) ara <sup>+</sup> lsi	(25)
<b><i>Escherichia coli</i></b>		
	Alc Complementation group	
8421	ara-1 Alc (CF isolate)	- (11)
8422	ara-1 alc <sup>+</sup> (spontaneous)	- (11)
8430	ara-1 Alc (stable)	- (21)
8490	ara-1 alc <sup>+</sup>	I (12)
8494	ara-1 alc <sup>+</sup>	I (12)
8587	ara-1 alc <sup>+</sup>	II (12)
8597	ara-1 alc <sup>+</sup>	III (12)
8674	ara-1 alc <sup>+</sup>	IV (11)
8693	ara-1 alc <sup>+</sup>	IV (12)
8638	ara-1 alc <sup>+</sup>	V (11)
8646	ara-1 alc <sup>+</sup>	V (11)
8657	ara-1 alc <sup>+</sup>	V (11)
8631	ara-1 alc <sup>+</sup>	VI (11)
8652	ara-1 alc <sup>+</sup>	- (11)
8640	ara-1 alc <sup>+</sup>	- (11)
8651	ara-1 alc <sup>+</sup>	- A. Dacine (11)
8655	ara-1 alc <sup>+</sup>	- (11)
8658	ara-1 alc <sup>+</sup>	- (11)
8663	ara-1 alc <sup>+</sup>	- (11)
8665	ara-1 alc <sup>+</sup>	- (11)
8667	ara-1 alc <sup>+</sup>	- (11)
8669	ara-1 alc <sup>+</sup>	- (11)
8674	ara-1 alc <sup>+</sup>	- (11)
8676	ara-1 alc <sup>+</sup>	- A. Dacine (11)
8678	ara-1 alc <sup>+</sup>	- A. Dacine (11)
<b><i>Plasmids</i></b>		
pC713	Km <sup>r</sup> Tc <sup>r</sup> lacP1 mob	(11)
pJAD215	Km <sup>r</sup> Sm <sup>r</sup> lacQ mob	(23)
pMB22	Ap <sup>r</sup> P <sub>lac</sub> lacQ/P4 mob <sup>+</sup> lac <sup>+</sup> lacI <sup>+</sup>	(46)
pMB24	Ap <sup>r</sup> P <sub>lac</sub> lacQ/P4 mob <sup>+</sup> lac <sup>+</sup> lacI <sup>+</sup>	(46)
pMB66H	Ap <sup>r</sup> P <sub>lac</sub> lacQ <sup>+</sup> lac <sup>+</sup> mob <sup>+</sup> lacI <sup>+</sup>	(30)
pMB66H	Ap <sup>r</sup> P <sub>lac</sub> lacQ <sup>+</sup> lac <sup>+</sup> mob <sup>+</sup> lacI <sup>+</sup>	(30)
pRK1313	Km <sup>r</sup> ColE1 mob <sup>+</sup> lac <sup>+</sup> (RK2)	(14)
pRKZ-1	Ap <sup>r</sup> promoter probe vector containing lacZ as reporter	A. Rothel (16)
pUC118	Ap <sup>r</sup> lacZ' M13 intergenic region	(45)
pUC119	Ap <sup>r</sup> polylinker of pUC118 inverted	(45)
pC21-1	Km <sup>r</sup> contains RNA polymerase gene	(16)
pT7-5	Ap <sup>r</sup> T7 promoter vector	(16)
pT7-6	Ap <sup>r</sup> T7 promoter vector	(16)
pHE6	Ap <sup>r</sup> P <sub>lac</sub> -lacZ transcriptional fusion	This paper
pAD1030	Ap <sup>r</sup> P <sub>lac</sub> -alcA (in plasmid pMB74)	(3)
pAB8	Tc <sup>r</sup> alc <sup>+</sup> cloned in pC713 (2.6 kb insert)	This paper
pHE15	Km <sup>r</sup> alc <sup>+</sup> cloned in pJAD215 (2.6 kb insert)	"
pHE15-Δ2C	Km <sup>r</sup> alc <sup>+</sup> cloned in pJAD215 (1.8 kb insert)	"
pHE15-ΔHE	Km <sup>r</sup> alc <sup>+</sup> cloned in pJAD215. P <sub>lac</sub> from (from pJAD215 removed (2.6 kb insert)	"
pHE15-50	Km <sup>r</sup> mutant alc <sup>+</sup> gene (alc <sup>+</sup> ) cloned in pJAD215 (2.6 kb insert)	"
pHE18	Ap <sup>r</sup> P <sub>lac</sub> -alc <sup>+</sup> cloned in pMB22 (2.6 kb insert)	"
pHE18	Ap <sup>r</sup> P <sub>lac</sub> -alc <sup>+</sup> cloned in pMB22 (1.8 kb insert)	"
pHE19	Ap <sup>r</sup> P <sub>lac</sub> -alc <sup>+</sup> cloned in pMB66H (1.8 kb insert, reverse orientation of pHE18)	"
pHE330	Ap <sup>r</sup> Km <sup>r</sup> alc <sup>+</sup> cloned in pT7-5 (same orientation as in pHE18)	"
pHE331	Ap <sup>r</sup> Km <sup>r</sup> alc <sup>+</sup> cloned in pT7-6 (same orientation as in pHE19)	"
<b><i>Phages</i></b>		
Miljop18	P <sub>lac</sub> lacZ	(46)
Miljop19	Miljop18 (polylinker inverted)	(46)
φ225	Ap <sup>r</sup> 1.5 kb HindIII-XbaI fragment of lacZ cloned in Miljop19	This study

an "r" superscript indicates resistance to Ap, Km, or Tc

## RESULTS

**Identification of an Alginate-negative mutant of *P. aeruginosa* lacking PPM activity.** Wang et al. (28) showed that none of the *P. aeruginosa* alg genes cloned previously in this laboratory encoded PPM. Thus we devised a strategy for cloning the *alg* gene based on the following results. St-Correlle et al. (31) showed that overexpression of the *P. aeruginosa* *algA* gene in *P. aeruginosa* leads not only to elevated levels of the *algA* gene product PHL-GDP, but also to elevated levels of PPM activity. The possibility that PPM was an activity of the hyperproduced PHL-GDP protein was eliminated, however, since PPM was easily separated from PHL-GDP by ion-exchange chromatography (31). Overproduction of the *E. coli* nonfunctional PHL (the product of the *E. coli* *algA* gene) in *P. aeruginosa* also led to elevated levels of PPM (31).

We exploited this inducibility of PPM to identify a mutant of *P. aeruginosa* that lacked PPM activity. Plasmid pAD4038 contains the *P. aeruginosa* *algA* gene cloned under control of the *lac* promoter in the broad host range vector pWD24 (31). pAD4038 was introduced (by triparental mating) into several alginase-negative mutants of *P. aeruginosa*, and the *algA* gene was overexpressed by IPTG induction. Crude extracts were prepared (from these cells), and the levels of PHL activity were determined. The extracts were then assayed for the concomitant increase in PPM activity that accompanies overproduction of PHL (31). Elevated levels of PPM activity were observed in all extracts except that prepared from mutant 8830 (Table II). Strains 8832/pAD4038 and 8836/pAD4038 were regrown and re-examined in a separate experiment. Both strains had the expected high levels of PHL and GDP, but again only 8832/pAD4038 had a concomitant increase in PPM activity (data not shown).

TABLE II

**Specific activities of PHL and PPM in crude extracts of alginase-negative mutants of *P. aeruginosa* overexpressing the *P. aeruginosa* *algA* gene (pAD4038)**

Strain	alg mutant	Specific activity (mU/mg)	
		PHL	PPM
8821	-	91.7	53.3
8822	-	234.5	31.4
8830	-	203.6	24.3
8890	algA1	283.3	36.6
8894	algA2	269.9	22.4
8887	algA3	212.7	34.4
8897	algA4	216.7	37.9
8874	algA5	298.3	32.5
8893	algA6	167.0	32.3
8836	algA7	310.7	32.4
8846	algA8	242.9	20.4
8857	algA9	68.3	22.0
8835	algA10	217.5	32.2
8832	algA11	118.4	34.0
8840	algA12	251.2	38.9
8851	algA13	116.1	30.6
8855	algA14	117.3	34.4
8858	algA15	116.7	41.0
8863	algA16	129.3	34.4
8865	algA17	271.1	32.9
8892	algA18	186.6	32.2
8895	algA19	68.6	26.2
8896	algA20	262.3	39.3
8898	algA21	206.6	31.3
8899	algA22	166.3	30.9

All of the alginase-negative mutants were derived from the stable succoid strain 8830 by EMS mutagenesis. The normal basal levels of PHL and PPM in succoid strains 8821 and 8830 (i.e., in the absence of plasmid-borne *alg* genes) are about 0.3-1.0 mU/mg and 2.0 mU/mg, respectively (31).

TABLE III

**Alginate production by *P. aeruginosa* strains**

Strain/plasmid	mg alginate/mg protein	
	24 h	48 h
8838	0	0.01
8838/pCP13	0.04	0.09
8838/pAB6	3.43	5.98
8830	4.17	7.84

pCP13 is the cloning vector used to construct pAB6.

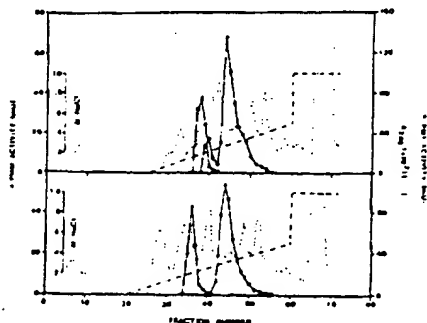


Fig. 3. Elution profiles of PHL (top) and PPM (bottom) from crude extracts of *E. coli* strains AC60 (bottom panel) and AC60/pst49 (+IPTG) (top panel). Fractions were fractionated by ion-exchange chromatography. The dotted and dashed lines represent A280 and the NaCl gradient, respectively.

1 2 3 4 5 6 7 8 9 10 11 12 13 14

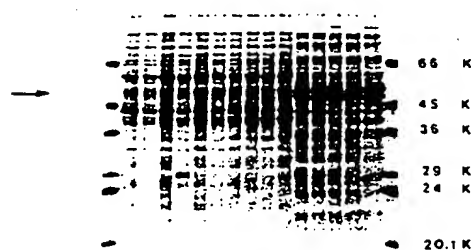


Fig. 4. SDS-PAGE of crude extracts of *P. aeruginosa* 8838 and *E. coli* AC60 harboring various plasmids containing the putative *P. aeruginosa* *algA* gene. Lanes with the appropriate vector and uninduced controls (ICAT to Table IV). Lanes are numbered as follows: 1, 8838; 2, 8838 (+IPTG); 3, 8838/pst22; 4, 8838/pst22 (+IPTG); 5, 8838/pst19; 6, 8838/pst19 (+IPTG); 7, 8838/pst666; 8, 8838/pst666 (+IPTG); 9, 8838/pst49; 10, 8838/pst49 (+IPTG); 11, AC60; 12, AC60/pst666; 13, AC60/pst666 (+IPTG); 14, AC60/pst49; 15, AC60/pst49 (+IPTG). Molecular weight markers are shown on both sides of the gel. The migration position of PPM (lanes 5, 6, 9, and 10) is indicated by the arrow.

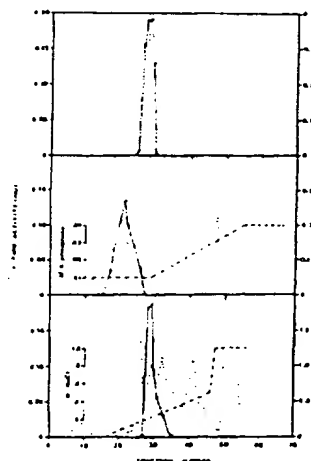


Fig. 5. Elution profiles of PPM (a) following sequential purification steps of ion-exchange chromatography (bottom panel), hydroxylapatite chromatography (center panel), and gel filtration chromatography (top panel). The dotted lines represent A280. The dashed line in the bottom and center panels represent the NaCl (ion-exchange) and potassium phosphate (hydroxylapatite) gradients.

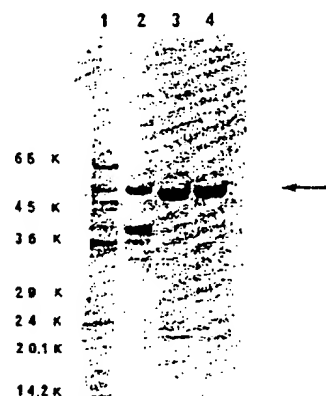


Fig. 6. SDS-PAGE of PPM preparations throughout purification steps (see Fig. 5). Lanes are numbered as follows: 1, crude extract; 2, ion-exchange; 3, hydroxylapatite; 4, gel filtration. Migration positions of molecular weight markers are shown on the left side of the gel. The arrow indicates the PPM band, migrating to a position corresponding to a molecular weight of approximately 31,000.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466
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107  
A I G C I I L T R S H N P G G P N G D F G I K PGN  
G K S G U N L T G S H N P P O Y N G F K I U PNN  
99

Fig. 2. Nucleotide sequence of the *algA* gene coding for YMW and the upstream flanking region. Nucleotides are numbered from the transcriptional start site of *algA* as +1. Both strands of the entire *algA* coding region and 421 bp upstream of the transcriptional start site are shown. The arrow indicates the direction of transcription and the transcriptional start site of the *algA* gene. The *algA* leader sequence is underlined. The translational initiation codon (ATG) is underlined. The sequence of the *algA* leader sequence is shown below the DNA sequence and is numbered with the translational initiation site as +1. The putative ribosome-binding site (GGAG) is indicated by (GGAG). The GC rich positions -11 and -22 and the GC rich positions -100 and -101 are indicated by (GC). The bases complementary to the primer used in 31 nucleases mapping are underlined. Bases shown in lower case letters (-86 to -94) are homologous to the sequence shown in the *algA* gene. The sequence of the *algA* protein is shown below the sequence of the *algA* gene [42].

CTT	Phi	0	TCY	ser	0	TAT	Arg	1	TCY	Cys	3
CTC	Phi	17	TCC	ser	6	TAC	Tyr	10	TGC	Cys	1
CTA	Leu	0	TCA	ser	0	TAA	End	0	TCA	End	1
CTT	Leu	1	CCY	ser	5	TAG	End	0	TGG	Tyr	5
CTT	Leu	3	CCY	Pro	1	CAT	His	1	CGT	Arg	6
CTC	Leu	3	CCC	Pro	7	CAC	His	5	CGC	Arg	19
CTA	Leu	0	CCA	Pro	0	CAG	Gln	1	CAG	Arg	0
CTT	Leu	14	CCY	Pro	18	CAG	Gln	10	CGG	Arg	1
ACT	Ile	1	ACT	Thr	2	AAT	Asn	2	ACT	Ser	0
ACT	Ile	10	ACC	Thr	19	AAC	Asn	14	AGC	Ser	14
ATC	Arg	0	ATC	Thr	0	AGA	Arg	0	AGC	Ser	0
ATC	Arg	8	ACC	Thr	1	AAG	Tyr	11	AGG	Ser	0
CTT	Val	0	GCT	Ala	2	CAT	Asp	6	GGT	Gly	4
CTC	Val	13	GCA	Ala	19	CAC	Asp	17	GCT	Gly	14
CTA	Val	4	GCA	Ala	1	CAG	Gln	8	GCA	Gly	0
CTT	Val	22	GCG	Ala	3	CAC	Gly	19	GCG	Gly	3

The ATG (Mgt) initiation codon is included